Unusual DNA Structure in the Regulatory Region of the Human Papovavirus JC Virus

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The human papovavirus JC virus (JCV) was analyzed for the presence of unusual DNA conformations. Recombinant plasmids containing 60% of the JCV prototype Mad-1 strain DNA were constructed and analyzed with both enzymatic and chemical probes. Fine-mapping studies revealed that the most prominent S1 nuclease-sensitive and bromoacetaldehyde-modified sites were located within the TATA boxes of each 98-base-pair tandem repeat. Further studies revealed that the S1 nuclease-sensitive site in the first TATA box (proximal to the origin) was ~50-fold stronger than the site in the second TATA box (distal from the origin). Deletion of the first TATA box drastically reduced the extent of bromoacetaldehyde modification in the second TATA box, whereas deletion of the second TATA box had little or no effect on the reactivity at the first TATA box. Hence, the biological and conformational role of the second TATA box remains unclear. No supercoil-induced relaxation was found, and reactions with the probes were not pH dependent. Also, fragments containing this regulatory region did not appear to be bent, although the A+T-rich segment contained a tract of eight consecutive A's. We conclude that the regulatory region of JCV contains non-B, but right-handed, DNA conformations which account for this behavior.

JC virus (JCV) is a human papovavirus which causes a fatal demyelinating brain disease, progressive multifocal leukoencephalopathy, in the brain of immunodeficient individuals (48). JCV is also an oncogenic virus since it induces a variety of tumors in hamsters (48, 68). It is also the only human virus known to cause brain tumors in primates (31, 42, 53). JCV also induces dysmyelination in transgenic mice (64). Although it is related genetically and antigenically to simian virus 40 (SV40) and the human BK virus, the biology of JCV infection is unique in that it shows host cell specificity, propagating well only in human fetal glial (PHFG) cells. JCV has a prolonged life cycle, requiring 3 to 4 weeks to produce significant viral titers in PHFG cells (38, 47). Because of the difficulties in propagating a sufficient amount of the virus in tissue culture, little is known about the molecular regulation of this virus. Recently, several cell lines have been characterized for propagating JCV in culture which were derived from PHFG cells (33, 34).

The genome of the Mad-1 strain of JCV has been sequenced (11, 13) and has extensive nucleotide sequence homology with SV40 and BKV DNAs (13). However, the greatest degree of nonhomology occurs in the regulatory regions of these three viruses. The regulatory region of the prototype JCV Mad-1 contains two 98-base-pair (bp) tandem repeats located in a position analogous to the 72-bp repeats of SV40 (9, 52) and the 68-bp repeats of BKV (57) which are known to be transcriptional enhancers (3, 18, 23). Each of the JCV 98-bp repeats contains the TATA box-like sequence TATATAT (28), a signal that positions the start site of early viral mRNA synthesis (3, 14, 15). The SV40 and BKV enhancer elements are located upstream of a single TATA box. Unlike SV40 and BKV, the JCV tandem repeats possess an enhancerlike function that is tissue specific for human fetal glial cells (21). In addition, the promoter-enhancer sequences of JCV variants isolated from different

patients with progressive multifocal leukoencephalopathy are reported to be hypervariable (37). The Mad-4 strain of JCV has a deletion of 19 bp which removes the second (distal) TATA box.

Because of recent studies on unusual non-B conformations in the DNA of tumor viruses such as SV40 (8, 43, 46, 56, 65), we investigated the structure of JCV DNA in this regulatory region. Conformational heterogeneity along the DNA strand has been demonstrated in several types of systems (reviewed in reference 71a). DNA, under certain conditions, may adopt non-B structures such as left-handed Z-DNA, which is found particularly in alternating purine-pyrimidine stretches (55, 70, 71), cruciforms which occur at inverted repeats (29, 50, 58), unusual structures at certain homopurine-homopyrimidine sequences (5, 8, 25, 51, 72), slipped structures, which were postulated to occur at direct repeats (32, 40), bent DNA, detected in fragments of kinetoplast DNA (36), SV40 (56), and other genetic control regions (4, 73, 75), and anisomorphic DNA that occurs in some direct repeat oligopurine-oligopyrimidine sequences (74). Negative supercoiling has been shown to stabilize all of these unusual structures except bent DNA.

S1 nuclease is a probe which can detect non-B-DNA structures such as left-handed Z-DNA, cruciforms, and slipped structures (32, 40, 50) and can react with functionally important regions such as the TATA box of the major late promoter of adenovirus (16, 25, 77) and the origin-enhancer region of SV40 (8). Treatment of DNA with chemical reagents such as bromoacetaldehyde (BAA) shows enhanced reactivity at unusually accessible bases along the DNA strand. When coupled with sequencing methods, BAA is useful for identifying regions of altered conformations. Probes for unusual DNA structures have been reviewed recently (69a). Recent studies on DNA tumor viruses with both S1 nuclease and chemical probes suggest that the secondary structures of DNA in the regulatory regions of the genome might play a role in the regulation of gene expression

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(8, 43). Studies by others have indicated structural heterogeneity in the vicinity of the origin of replication (56, 65).

Hence, we conducted a study on portions of JCV to determine the presence of unusual DNA structures which may explain its unique biological behavior. Enzymatic, physical, and chemical probes were used to define and correlate the potential regions of altered conformations with sites important for genetic expression. Recombinant plasmids containing 60% of the genome were constructed and studied. We found unusual non-B DNA structures in the TATA boxes of JCV Mad-1 strain. Studies were also conducted on two sets of plasmids harboring deletions in the first and second TATA boxes. Deletion of the first TATA box affected the presence of these structures, while deletion of the second TATA box had no effect on the altered structure. The non-B conformation was different from Z-DNA, cruciforms, anisomorphic DNA, and slipped structures.

MATERIALS AND METHODS

Recombinant plasmids. JCV genomic DNA was prepared from the plasmid pMad-1TC (12), which contains the entire genome of the prototype JCV Mad-1 strain inserted into the *EcoRI* site of pBR322. To isolate the JCV genome from the vector, the plasmid was digested with *EcoRI* and *HhaI* into 31 fragments of pBR322 (there are no *HhaI* restriction sites in JCV Mad-1). When passed over a Sephacryl S-1000 column (Pharmacia), the 5.1-kbp JCV fragment was eluted in the void volume, whereas the pBR322 fragments were retarded in the Sephacryl beads. The JCV DNA was recovered by precipitation and suspended in TE buffer (10 mM Tris hydrochloride, pH 7.5, 0.1 mM EDTA). Restriction enzyme and sequence analyses proved the presence of the JCV Mad-1 DNA. This DNA was used to construct recombinant plasmids representing both coding (late and early) and non-coding regions (regulatory region) of JCV Mad-1. The recombinant plasmids used in this study represent 60% of the JCV genome. The JCV genome (Fig. 1) was digested with *MboI*, and the following fragments were isolated and cloned into the *BamHI* site of pBR322. Fragment A (pJC661) is a 661-bp fragment (nucleotides 2078 to 2739) of JCV Mad-1 which contains the overlapping 3' ends of the early and late mRNAs. Fragment B (pJC529) is a 529-bp fragment (nucleotides 2739 to 3268). Fragment C (pJC396) is a 396-bp fragment (nucleotides 3343 to 3739). Fragments B and C contain part of the early region of JCV Mad-1 which codes for large T antigen (Fig. 1). Fragment E (pJC421) is a 421-bp fragment (nucleotides 970 to 1391) of the late region which codes for viral proteins VP2 and VP3. The recombinant plasmids were transformed into *Escherichia coli* HB101. Clones were screened by restriction analyses to ascertain their orientations. To study the regulatory region (fragment D) of JCV, two plasmids were constructed. pJC286 contains the 286-bp *HindIII-PvuII* fragment of the regulatory region of the JCV Mad-1 strain (0.67 to 0.72 map units). This region was originally cloned into the *BamHI* site of the M13 vector mp18 by using *BamHI* linkers. The 286-bp fragment of the Mad-1 regulatory region was excised from the M13 clone with *BamHI* and subcloned into the *BamHI* site of pBR322. pJC120 is a derivative of pJC286 which contains only 98 bp of the regulatory region of JCV Mad-1. To construct this plasmid, pJC286 was digested with *SstI*, and the 98-bp fragment (nucleotides 57 to 155), containing the second but not the first TATA box, was cloned into the *SstI* restriction site within the polylinker of pUC18 to give pJCpUC98. A

120-bp fragment was then excised from this clone by using the *EcoRI* and *BamHI* sites on either side of the 98-bp fragment within the polylinker, purified, and inserted between the *EcoRI* and *BamHI* sites of pBR322. (pBR322 sequences between the unique *EcoRI* and *BamHI* restriction sites had been deleted.) Colonies resulting from the transformation of *E. coli* HB101 were screened for the presence of the inserts by using various restriction endonucleases. The regulatory region of JCV Mad-4 (37), a variant of Mad-1 which has a deletion of 19 bp that removes the second TATA box (distal from the origin), was isolated from pM4 3'scat. pM4 3'scat contains the regulatory region of JCV Mad-4 cloned into the *BamHI* site of vector pA₁₀cat₂ (27). pA₁₀cat₂ contains the *SphI-BamHI* fragment of pSV2cat inserted into the corresponding site of pA₁₀ (a pBR322 derivative plasmid with a deletion from *BamHI* to *EcoRI*). To study the regulatory region of SV40, pSVA2 was constructed from pSV2cat vector. pSV2cat has been described (17) and contains the SV40 enhancer and the TATA box linked to the bacterial gene coding for chloramphenicol acetyltransferase (CAT). pSV2cat was digested with *EcoRI*, and the resultant 2.8-kbp *EcoRI* fragment containing the *EcoRI-PvuII* fragment of pBR322 and the regulatory region (*HindIII-PvuII*) of SV40 linked to the 252-bp fragment of the bacterial *cat* gene was recircularized by ligation. The ligation mixture was used to transform *E. coli* HB101, and ampicillin-resistant colonies were screened for the presence of the 340-bp fragment from the SV40 regulatory region.

BKV plasmid DNA (Dunlop strain) was kindly provided by N. Rosenthal (Massachusetts Children's Hospital Medical Center) and contains the entire BKV genome cloned into the *BamHI* site of pBR322. All the clones were screened by

FIG. 1. General map of JCV Mad-1 (13). The unique *EcoRI* site is taken as 0 or 1.0 map position. The origin of DNA replication (ORI) is located at 0.67 map units. Arrows extending from the origin indicate the direction of transcription. The sequence (5' to 3') starting counterclockwise after the origin represents the early region which codes for small t and large T antigen. The sequence (5' to 3') clockwise from the origin represents the late region which codes for agnoprotein and VP2, VP3, and VP1 proteins. The heavy lined areas indicate the parts of the genome which were cloned into the *BamHI* site of pBR322 as described in Materials and Methods.

restriction analyses to prove their orientations. The inserts were characterized by Maxam-Gilbert sequencing (39).

Nuclease reactions. Nuclease reactions were performed essentially as described previously (60). S1 and P1 nucleases were obtained from Bethesda Research Laboratories. Plasmid DNA (1.5 μ g) was treated with S1 nuclease (2.0 U) at 37°C for 30 min in 50 μ l of S1 nuclease buffer (40 mM sodium acetate [pH 4.6], 50 mM NaCl, 1 mM ZnSO₄). Reactions were terminated by addition of EDTA to a final concentration of 30 mM. The DNA was dialyzed against the restriction enzyme buffer for 2 h and digested with appropriate restriction enzymes. The DNA fragments were then separated by agarose gel electrophoresis. P1 nuclease reactions were carried out in a solution containing 10 mM Tris hydrochloride, pH 7.5, 50 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, and 1 U of P1 nuclease per 1.5 μ g of DNA for 1 min at 37°C. Reactions were stopped by adding EDTA to a final concentration of 30 mM. The DNA was dialyzed against restriction buffer, digested with appropriate restriction enzymes, and analyzed by agarose gel electrophoresis to locate the P1 cleavage sites.

Fine mapping of S1 nuclease-nicked sites. Supercoiled DNA (density, -0.05 to 0.06) was digested with S1 nuclease as described above except that 20 U of enzyme per 1.5 μ g of DNA was used for 1 min at 37°C. After digestion with appropriate restriction enzymes, the DNA fragments were either 5'-end labeled with T4 polynucleotide kinase or 3'-end labeled with the Klenow fragment of *E. coli* DNA polymerase (Boehringer-Mannheim) by established procedures. Following digestion with a second restriction enzyme, the labeled fragments were separated on polyacrylamide gels. The positions of the S1 nuclease-specific nicks were then determined by electrophoresis on denaturing polyacrylamide gels alongside Maxam-Gilbert sequencing ladder markers. Autoradiograms of the gels were scanned with a Bio-Rad model 620 video densitometer to quantitate the bands.

BAA reactions. BAA was prepared as described (74; M. J. McLean, J. E. Larson, F. Wohlrab, and R. D. Wells, *Nucleic Acids Res.*, in press). For BAA reactions, 3.0 μ g of DNA in 100 μ l of 10 mM Tris hydrochloride (pH 7.5)-50 mM NaCl-10 mM MgCl₂-1 mM dithiothreitol was incubated at 37°C for 3 h in the presence of 1% BAA. The DNA was extracted three times with ether, precipitated with ethanol, and dissolved in restriction enzyme buffer. Digestion was then carried out with the appropriate restriction endonuclease. A total of 15 μ l of S1 nuclease buffer containing 1.5 U of S1 nuclease was then added to this solution and incubated for 10 min at 37°C. The reactions were terminated by addition of EDTA (pH 8), and the DNA fragments were separated by agarose gel electrophoresis. The BAA-sensitive sites were mapped as previously described (19; McLean et al., in press). For fine mapping of BAA-modified sites, the supercoiled DNA was treated with BAA as described above. After digestion with the appropriate restriction enzymes, the DNA fragments were 3'-end labeled with the Klenow fragment of *E. coli* DNA polymerase by established procedures. The labeled fragments were separated on polyacrylamide gels, and the fragments of interest were eluted and purified. The DNA fragments were then treated with 20 U of S1 nuclease in 20 μ l of S1 nuclease buffer for 2 min at 37°C and recovered by precipitation. The location of BAA-modified sites was determined on polyacrylamide sequencing gels alongside Maxam-Gilbert sequencing ladder markers.

Supercoil relaxation studies. Topoisomeric samples of the plasmids were generated with ethidium bromide and calf thymus topoisomerase I (gift of J. E. Larson) as described

(63). The superhelical density of each population was determined by fluorescence enhancement due to ethidium bromide binding to DNA in a Perkin-Elmer spectrofluorimeter. Two-dimensional gel electrophoresis was performed essentially as described (19, 41, 69, 74). Other materials and methods were as described previously (19, 24, 25, 41, 60-63, 74).

RESULTS

Construction of recombinant plasmids containing fragments of the JCV Mad-1 genome. To analyze unique structural properties of the JCV genome, we constructed recombinant plasmids with pBR322 as a vector representing 60% of the JCV DNA. Four recombinant plasmids were constructed which contained both early and late regions of the JCV Mad-1 strain. Figure 1 shows a general map of JCV Mad-1 (13). The regions of JCV designated by heavy dark lines are the fragments used in this study. JCV Mad-1 was digested with *Mbo*I, and the corresponding fragments were cloned into the *Bam*HI site of pBR322. To study the regulatory region of JCV Mad-1, pJC286 was constructed, which contains a 286-bp *Hind*III-*Pvu*II fragment (nucleotides 5112 to 268) embodying the entire regulatory region of JCV Mad-1 (Fig. 2). As shown, pJC286 contains two 98-bp tandem repeats with two TATA boxes. Vertical arrows indicate the position of two *Sst*I sites (nucleotides 57 and 155) on the tandem repeats. Since our studies on JCV indicated the presence of unusual DNA conformations in the TATA boxes, it was important to investigate the effect of neighboring sequences. Therefore, pJC120 was constructed (Fig. 2), which contains a 98-bp fragment of pJC286 which eliminates the first (proximal to the origin) TATA box. Studies were also conducted on the regulatory region of JCV Mad-4 (37), a variant of JCV Mad-1 which has a deletion of 19 bp that removes the second (distal from the origin) TATA box (Fig. 2). The segment studied was a 268-bp *Hind*III-*Pvu*II fragment of JCV Mad-4 which contained the entire control region of Mad-4 strain of JCV. This fragment was isolated from the vector pM4 3'scat as described in Materials and Methods.

Studies were also conducted with other papovaviruses. The genetic organization of JCV has great similarities with SV40 and human BKV (66). JCV has 70 to 80% nucleotide homology with SV40 and BKV (13). Comparative studies were also conducted on the regulatory regions of SV40 and BKV, since this region shows the greatest divergence between these viruses. The regulatory region of SV40 was studied by constructing pSVA2 from the vector pSV2cat. The control region of BKV was isolated from the parental vector containing the entire BKV Dunlop strain (57) inserted into the *Bam*HI site of pBR322.

BAA reactions. BAA is a sensitive probe for detecting unpaired loops of cruciform structures (19, 30) and B-Z junctions (McLean et al., in press), reacting with A and C residues to give imidazole derivatives (20), which cannot base pair (reviewed in Wells et al., in press). The location of these "wedged-open" regions can be mapped with S1 nuclease after linearization of the plasmids. We used BAA as a probe to evaluate the presence of unusual structures in the JCV genome. Negatively supercoiled pJC286 was reacted with BAA and linearized with *Bam*HI-*Sst*I. The positions of the modified sites were determined by S1 nuclease cleavage of the linearized plasmid. Restriction mapping assays revealed the presence of BAA-modified sites in the insert (data not shown). This modified site was mapped more precisely at

FIG. 2. Inserts in plasmids containing the regulatory region of JCV. pJC286 contains the *Hind*III-*Pvu*II fragment of JCV Mad-1. Shown from left to right are the origin of replication (ORI), the two 98-bp tandem repeats (enhancer regions), and the two A+T-rich TATA-like elements (AT) located within the 98-bp repeats. The TATA box on the left in pJC286 is referred to as the first TATA box, and the one on the right is the second. The 286-bp fragment of the control region was cloned into the *Bam*HI site of pBR322 as described in Materials and Methods. pJC120 is an *Sst*I fragment of pJC286 which eliminates the first TATA box (proximal to the origin) and contains the 98-bp fragment (nucleotides 57 to 155) to JCV Mad-1 plus an additional 22 bp of the pUC18 polylinker sequence (i.e., *Eco*RI to *Bam*HI). The 120-bp fragment was cloned into the *Eco*RI-*Bam*HI site of pBR322 as described in Materials and Methods. pJCMad-4 has a deletion of 19 bp which removes the second TATA box (distal from the origin) at nucleotides 110 to 129. The arrows indicate the positions of *Sst*I sites (nucleotides 57 and 155) in both 98-bp tandem repeats of JCV. The triangle underneath the second tandem repeat of pJCMad-4 indicates deletion of the second TATA box.

the base-pair level on both strands. Figures 3a and b show the BAA fine mapping of both strands of pJC286. The major modified sites observed mapped to the first and second TATA boxes. The strong site in the first TATA box was near nucleotide 20. The modified sites in the second TATA box spanned a 30-bp region, nucleotides 110 to 140, with a strong site at 125. The breadth of these bands was due to the presence of several BAA reaction sites within these regions. Additional sites were observed at nucleotides 100 and 195 (also see Fig. 6), which mapped near the putative enhancer core sequence 5'-GTGCTTTG-3'. These sites are within the region known as the JCV late RNA 5' start sites (21). The BAA-sensitive sites at the 5'-TACCTA-3' sequences at positions 64 and 162 were interesting from a different perspective. Their locations at ~30 bp upstream from the two late RNA 5' start sites were analogous to the similar sequence found in SV40 upstream of the major late RNA start site. In SV40, this sequence has been characterized as a surrogate TATA box that controls the level of RNA synthesis (45). Its function, however, in JCV as a late promoter has not been characterized (21).

Similar studies were also conducted on four other plasmids containing other segments of the JCV genome; pJC661 (fragment A), pJC529 (fragment B), pJC396 (fragment C), and pJC421 (fragment E). The results indicated the presence of some weak sites in all plasmids studied. pJC661 showed an additional site at nucleotide 2150 within the sequence 5'-GTATATTG-3'. In summary, we conclude that the most

prominent BAA-modified sites are at the first and second TATA box of pJC286 (fragment D).

S1 nuclease-hypersensitive sites in the JCV genome. S1 nuclease is a useful tool in detecting several types of non B-DNA structures (see Introduction). Supercoiled plasmids were treated with S1 nuclease for 1 min so that the majority of molecules were nicked, as described in Materials and Methods. Restriction endonuclease mapping of double-stranded S1 nuclease cleavage sites were conducted on supercoiled plasmids at native densities (-0.05 to 0.06), which revealed the presence of insert-specific sites. Fine-mapping studies were conducted on the S1 nuclease-treated plasmids to determine the number and positions of the initial cleavage sites at the base-pair level. For all of the plasmids containing segments of JCV, the most prominent nicking sites were found in pJC286. Figure 3c shows fine mapping of the S1 nuclease cleavage sites in supercoiled pJC286 DNA. Interestingly, the major nicking site was found within the first TATA box. As discussed below, the two TATA boxes

FIG. 3. BAA and S1 nuclease reactions on pJC286. (a) Fine mapping of BAA-modified sites of the bottom strand of pJC286. Supercoiled plasmid was treated with BAA as described in Materials and Methods. The BAA-modified DNA was digested with *Bma*HI and *Sst*I. The 75-bp fragment containing the first TATA box (proximal to the origin) was then 3'-end labeled at the *Bam*HI site. The fragment was purified from a 5% polyacrylamide gel. After treatment with S1 nuclease, the fragment was analyzed on a 12% polyacrylamide-8 M urea gel alongside Maxam-Gilbert sequencing markers. (b) Fine mapping of BAA-modified sites of the top strand of pJC286. The plasmid DNA was treated with BAA. The BAA-modified DNA was digested with *Bam*HI and *Hae*III. The 276-bp fragment containing both 98-bp tandem repeats was then 3'-end labeled at the *Bam*HI site as described in Materials and Methods. After treatment with S1 nuclease, the fragment was analyzed on an 8% polyacrylamide-8 M urea gel alongside Maxam-Gilbert sequencing markers. (c) Fine mapping of the S1 nuclease cleavage sites of pJC286. Supercoiled DNA was treated with S1 nuclease as described in Materials and Methods. After dialysis, the plasmid was digested with *Bam*HI. The linearized DNA was then 5'-end labeled with T4 polynucleotide kinase. Following the second digestion with *Hae*III, the 276-bp fragment containing both 98-bp tandem repeats was analyzed for the location of the S1 nuclease-specific nicks by electrophoresis on a 5% denaturing gel alongside Maxam-Gilbert sequencing markers. The second TATA box was located at ~40% of the length of this gel.

responded quantitatively in a different manner. Densitometric scanning revealed that the S1 cleavage site in the first TATA box was approximately 50-fold stronger than the site in the second TATA box. This quantitative analysis is of significance, since the 15-nucleotide A+T-rich segment with identical sequences within each repeat responded differently to S1 nuclease. The difference in the structures of these TATA boxes may be due to the different environments resulting from sequences flanking the early end of these A+T-rich regions.

In addition to this major site, there were three weak sites visible after long exposure with equal intensity around the enhancerlike sequences at nucleotide 105 in the first 98-bp tandem repeat and at nucleotides 193 and 211 in the second repeat. Two sites with medium intensity were found in the pyrimidine-rich sequences in the second tandem repeat at nucleotides 123 and 131, which are within the regions of JCV late RNA 5' start site, and a site at nucleotide 70 with medium intensity which mapped at 5'-ATGGAT-3'. The nature of these sites was discussed above.

These sites are in excellent agreement with the sites recognized by BAA. The site in the first TATA box of pJC286 was the most sensitive to S1 nuclease found for any of the plasmids containing regions of JCV.

Effect of flanking sequences on formation of the unusual structures. To investigate the nature of the nucleotide sequences required to generate the BAA modification sites in the TATA boxes, we constructed pJC120, which has a deletion of the first TATA box. Negatively supercoiled pJC120 was treated with BAA, digested with *EcoRI* and *KpnI*, and 3'-end labeled at the *EcoRI* site as described earlier. Removal of the first TATA box substantially decreased the BAA reactivity at the second TATA box (Fig. 4a). Also, it shifted the site toward the early and late sides of the TATA box. The two remaining bands at nucleotides 110 and 130 were the same as those observed for pJC286 (Fig. 3b).

Studies were also conducted on the Mad-4 strain of JCV. Figure 4b shows the results of BAA fine mapping of pJCMad-4. The most prominent band observed mapped to the first TATA box. Together with analyses on the complementary strand (not shown), these experiments revealed that the major BAA-modified site was at nucleotide 15 in the first TATA box. In addition, there were two sites with medium intensity at nucleotides 130 and 105 (see Fig. 6) which mapped near the enhancer core sequence, plus a fairly strong site at nucleotide 70 (5'-ATGGAT-3') as observed in pJC286.

pJC120 and pJCMad-4 were also subjected to S1 nuclease nicking experiments. Figure 4c shows the S1 nuclease fine mapping of pJC120. As expected, no S1 nuclease site was present in the second TATA box. Figure 4d shows the results of S1 nuclease fine mapping of pJCMad-4. The major modified site was found to be around the TATA box, with a shift towards the end of eight consecutive A residues. In addition, there were two common sets of bands at nucleotides 35 and 45 around the sequence 5'-GGGAAGGG-3', plus a weaker band at nucleotide 70, as observed in pJC286.

Effect of pH on nuclease recognition. Similar studies were performed on supercoiled pJC286, pJC120, and pJCMad-4 with P1 nuclease. P1 nuclease has been used as a probe for unusual structures at neutral pH. This enzyme has similar properties to S1 nuclease, but functions at neutral pH and lacks the requirement for exogenous zinc. pJC286, pJC120, and pJCMad-4 were treated with P1 nuclease at pH 7.5 as described in Materials and Methods. The sites of cleavage on

FIG. 4. Fine mapping of BAA-modified sites and S1 nuclease nicks in pJC120 and pJCMad-4. The plasmids were treated with BAA (a and b) or S1 nuclease (c and d) as described in Materials and Methods. (a) pJC120. The BAA-modified DNA was digested with *EcoRI* and *KpnI* and then 3'-end labeled at the *EcoRI* site. (b) pJCMad-4. The BAA-modified DNA was digested with *BamHI* and *HaeIII* and then 3'-end labeled at the *BamHI* site. After treatment with S1 nuclease, the fragments were analyzed on a denaturing polyacrylamide gel alongside Maxam-Gilbert sequencing markers. A control lacking BAA revealed the authentic nature of the BAA-modified sites except for the full-length band and the site at nucleotide 30 in the purine-rich sequences flanking the first TATA box and two sites at nucleotides 70 and 165. The nature of these sites has been discussed previously. (c) Fine mapping of S1 nuclease nicks in pJC120. pJC120 was digested with *EcoRI* and *KpnI* and then 3'-end labeled at the *EcoRI* site. (d) Fine mapping of S1 nuclease nicks in pJCMad-4. pJCMad-4 was digested with *BamHI* and *HaeIII* and 3'-end labeled at the *BamHI* site. The S1 nuclease-specific nicks were determined on a denaturing polyacrylamide gel alongside the sequencing markers.

both strands were identical to those observed with S1 nuclease (data not shown). Thus, protonation does not seem to be necessary for stabilization of the alternate DNA structure(s) in the regulatory region of JCV.

Effect of supercoiling. The influence of negative supercoiling on BAA-modified sites in pJC286 was investigated. Fine-mapping studies were performed on negatively supercoiled, relaxed, and linear DNA (Fig. 5). The most striking observation from this set of experiments was that the profiles for the supercoiled (lane a) and relaxed (lane b) molecules were identical, with slightly lower intensity of the modified sites in the relaxed form. When pJC286 was linearized prior to the BAA reaction (lane c), a smear of low-intensity sites was observed, presumably due to BAA reaction initiated at the ends of the 82-bp fragment. Very little or no specific reaction at the TATA box was found, consistent with the results of other restriction mapping studies (not shown). This implies that a small amount of torsional strain is required for BAA reactivity in this region. A preparation of relaxed topoisomers consists of a distribution with positive and negative supercoils around the relaxed isomer, which has zero linking difference. Presumably, even the small amount of supercoiling present in the relaxed preparation was sufficient to elicit the conformations giving the reactions described above. Furthermore, a preparation of topoisomers with an average negative density of 0.03 elicited reactions similar to that seen in Fig. 5, lane a (not shown). The lack of supercoil-induced relaxation (see below) is consistent with these conclusions.

FIG. 5. Fine mapping of BAA-modified sites in supercoiled, relaxed, and linear pJC286 DNA. Lane a, Negative supercoiled DNA. Lane b, Relaxed DNA. Supercoiled DNA was relaxed with topoisomerase as described in Materials and Methods prior to treatment with BAA. For lanes a and b, the modified DNAs were digested with *Bam*HI and *Sst*I. Lane c, Linear DNA. Supercoiled DNA was linearized with *Bam*HI-*Sst*I prior to treatment with BAA. Following labeling at their 3' ends, in all cases at the *Bam*HI sites, the 75-bp fragments were purified from polyacrylamide gels. The BAA-reactive sites were cleaved with S1 nuclease, and the fragments were electrophoresed on a denaturing polyacrylamide gel alongside Maxam-Gilbert sequencing markers.

Two-dimensional gel studies. Supercoiling relaxation studies provide a critical analysis for the presence of underwound sequences (24, 60, 69, 74). Two-dimensional gel electrophoresis was used to analyze the topoisomers of the plasmids shown in Fig. 2. Mixtures of topoisomer populations of pJC286, pJC120, and pJCMad-4 were subjected to two-dimensional agarose gel electrophoresis as described in Materials and Methods. In all cases, no relaxation of the topoisomers was observed (data not shown) in comparison to the pBR322 control, indicating that the structural feature present in the regulatory region is not due to left-handed Z-DNA, cruciform DNA, or anisomorphic DNA. Similar experiments were conducted on pJC661, pJC529, pJC421, and pJC396. In all cases, no supercoil relaxation of the topoisomers was observed.

Assay for bent DNA. Bent DNA has been identified in DNA fragments from kinetoplasts and other genetic control regions (4, 35, 75) and in region 1 of the binding site of the SV40 large T antigen (6, 56). Intrinsic bending of DNA occurs at the junction of homopolymeric dA · dT with adjacent B-DNA (26, 73). The bending of DNA leads to decreased mobility during electrophoresis through polyacrylamide gels of appropriate porosity. Three linear DNA fragments of 286, 500, and 600 bp containing the T-antigen-binding site of pJC286 were analyzed for the presence of bent DNA. No unusual gel mobilities were observed (data not shown) in three different gel concentrations (12, 15, and 20%) at 4°C and room temperature, indicating the absence of a detectable bend in this region.

DISCUSSION

Treatment of the JCV genome with BAA and S1 nuclease revealed sensitive cleavage sites predominantly in the non-coding region, a segment that carries the regulatory signal for JCV early and late transcription, including the transcriptional enhancers. Such sensitivity indicates the presence of unusual DNA structures. The most sensitive sites were found in part of the JCV genome which correlated with the regions important for control of gene expression, namely the JCV late RNA 5' start sites (21) and the TATA box, which also influences the level of transcription and positions the cap site of the mRNAs (3, 14, 15). Our results with pJC286 clearly demonstrate that the strongest sites were within the TATA box located in each tandem repeat.

A number of explanations have been given for the presence of S1 nuclease-sensitive sites in supercoiled DNA molecules. These include junctions between left-handed Z-DNA and right-handed B-DNA (24, 41, 60, 61), cruciform structures at inverted repeats (29, 50, 61, 62), homopurine-homopyrimidine stretches (5, 25, 51), and anisomorphic DNA (74). It was suggested that the S1 nuclease cleavage site in the TATA box of the major late promoter of adenovirus is due either to a purine-pyrimidine bias (25) or to strand slippage at the direct repeats flanking the TATA box (77). Inspection of the nucleotide sequences of the region surrounding the S1 nuclease nicking site at the TATA boxes of JCV Mad-1 revealed the absence of these features. Furthermore, since no supercoil relaxation was observed, left-handed Z-DNA, cruciform structures, and anisomorphic DNA can be ruled out.

The sites cleaved by S1 nuclease and modified with BAA occurred on both strands, and the absence of a staggered reaction pattern excludes the presence of slipped structures (32, 40, 77). Furthermore, putative slipped structures do not seem to react with BAA (19, 41a). The negative superhelical density required to induce the altered structure within the TATA boxes of JCV was very low. Similarly, a negative superhelical density of only 0.02 to 0.03 has been observed for the S1 nuclease cleavage of the TTAA region of crab satellite DNA. However, in this case, the altered structure was strongly pH dependent (10), which is in contrast to the JCV Mad-1 sequence. P1 nuclease at neutral pH showed a fine-mapping profile in the TATA box similar to that found with S1 nuclease. Assuming that the S1 and P1 nuclease conformational probes recognize similar features, these results seem to exclude the protonation of C residues (51) as the basis of the formation of this altered structure (Fig. 6).

Poly(dA) · poly(dT) tracts possess an altered structure that may lead to bending of A tracts relative to adjacent B-DNA (1, 2, 75). The replication origins of papovaviruses have an A+T-rich segment containing a continuous run of eight adenine residues adjacent to the T-antigen recognition sites (66). This region in SV40 has been shown to have a conformation which is characteristic of DNA bending (6, 56). The sequences surrounding the first TATA box of JCV Mad-1 have close similarities to those of the SV40 core origin of replication. We examined several fragments of the core origin of JCV Mad-1 containing the AT segment for the presence of DNA bending. This region of JCV Mad-1 did not cause any detectable mobility shift in our polyacrylamide gel electrophoresis assay. Computational analyses with the "ApA" wedge model (67) were also consistent with our electrophoretic results, indicating that, contrary to SV40, there is no major bend in the core origin region of JCV.

The nature of the non-B-DNA conformation is unknown

FIG. 6. Schematic representation of the S1 nuclease cleavage and BAA-modified sites in two 98-bp tandem repeats of pJC286. Vertical lines indicate the positions of these sites. The length of each line reflects the intensity of the corresponding band on the autoradiograms, quantitated as described in Materials and Methods. Minor sites are denoted by dots, although they were not equal in intensity. The upper strand, read left to right, has the polarity and sequence of late mRNAs. The 98-bp repeats are indicated. Nucleotide numbering is as described previously (13).

but is none of the previously described unusual DNA structures. Presumably, an altered stacking of base pairs in a right-handed helix elicits the reactivities described here.

The BAA fine mapping of the regulatory region of SV40 and BKV revealed a pattern of multiple weak sites which mapped to the various functional elements in this region. These include the GC box 5'-GGGCGG-3', a binding site for the transcription factor Sp1 (7), and the T-antigen-binding sites 5'-GAGGC-3'. In contrast to JCV, no BAA-modified sites were observed within the TATA box of SV40 and BKV. The major site observed here mapped in the spacer region of the late side of the A+T-rich segment (6). These modified sites may be due to the presence of several functional elements in this region of SV40 and BKV which are not present in JCV, namely the Sp1-binding sequences.

JCV Mad-1 contains two 98-bp tandem repeats which function as enhancers (22). The duplication of TATA sequences and their location inside the tandem repeats represent a unique feature of JCV among the polyomaviruses. What is the role of these TATA boxes and what effect might they have on the altered structure? Our data on pJC286 showed that the S1 nuclease cleavage site in the first TATA box was approximately 50-fold stronger than the one in the second TATA box. To explain this behavior and to study the effects of these TATA boxes on the altered structure, we conducted similar experiments with the Mad-4 strain of JCV. A major BAA-modified site appeared to be within a long A+T-rich stretch near the first TATA box, indicating that removal of the 19 bp in JCV Mad-4 (second TATA box) did not affect the altered structure within the first TATA box. The fact that the second TATA box in JCV Mad-1 showed reactivity with BAA and S1 nuclease indicates that the two TATA boxes within the tandem repeats influence each other.

Kenney et al. (22) have shown that both tandem repeats of JCV are necessary to give full enhancer activity in CAT expression assays. In JCV Mad-4, deletion of the second TATA box did not eliminate the reactive sites in the first TATA box, nor did it lower its CAT activity significantly (D. Vacante, personal communication). This finding was not surprising, particularly since several viable variants of JCV have been isolated which have deletions of the second TATA box in their tandem repeats (37). These results clearly indicate that both tandem repeats and the first TATA box of JCV are necessary to give full CAT activity and that the deletion of any of these elements will result in loss of CAT activity.

The function of the second TATA box is not clearly known, but its absence in the JCV Mad-4 strain and other JCV isolates and the fact that it has not been found in the late region of BKV and SV40 (9, 44, 57, 76) suggest that it is not required for late transcription (13). Thus, our results and those of others seem to indicate that the second TATA box of JCV is not required either in vivo (clinically) or in vitro, as observed from its structural analysis and its CAT activity.

Of particular interest were experiments in which the first TATA box was removed from the other repeat sequences. Deletion of the first TATA box had a more drastic effect on the altered structure. Thus, its absence eliminated the S1 nicking sites in the second TATA box. The strength of the BAA-modified sites was also decreased. This was expected, since our results clearly indicated the importance of the first TATA box. pJC120 lacks the first TATA box, and since the second TATA box appears to be functionally unimportant, we would not expect to see an altered structure in this construct. Based on these results and the fact that there were no JCV isolates which resembled pJC120, we would predict that pJC120 will not show any CAT activity.

Our structural analyses indicated the presence of an altered structure in the TATA box of the first tandem repeat of JCV. Reynolds et al. (54) observed that the antitumor antibiotic CC-1065 cuts in the TATA region of SV40. It is interesting that the proximal TATA box of JCV lies in a position analogous to the TATA box of SV40. On examination of the sequences of both tandem repeats of JCV, two differences are apparent. (i) The first tandem repeat contains flanking sequences not present in the second repeat. Since the distribution of purine-pyrimidine residues between the two strands of each tandem repeat was not altered in all JCV constructs, the altered structure that is recognized by S1 nuclease and BAA is not simply a result of homopurine-homopyrimidine sequences flanking these TATA boxes. Moreover, the synthetic (dA)₂₀ · (dT)₂₀ in a plasmid is not reactive with BAA (J. C. Hanvey, personal communication). (ii) The 98-bp tandem repeats differ in sequences flanking the early side of both the TATA boxes. A cluster of pentanucleotides, 5'-GAGGC-3', is located at the early end of the first TATA box. The papovaviruses control their own replication through the interaction of viral T antigen with these pentanucleotide sequences (66). Mutations in this region resulted in a decrease of replication in SV40 (6). It also has been suggested that DNA bending may be a structural signal for DNA replication in papovaviruses (6). Although our results did not show a major bend in this region of JCV DNA, they do not exclude the possibility of protein-induced bends near the conserved A tract. One example of protein-induced bending is the case of the binding of lambda O protein to the lambda origin of replication (78).

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